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RADIATION-INDUCED ALTERATIONS IN SERUM AND SPLENIC LYSOSOMAL HY--ETC(U)
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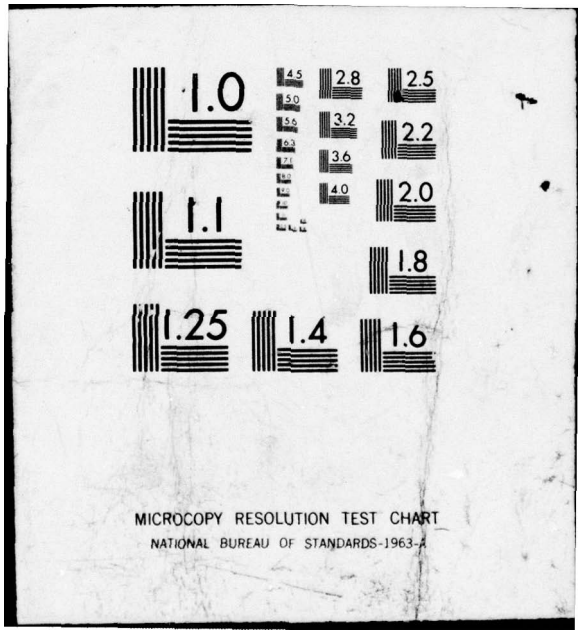
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Exposure to ionizing radiation may result in periods of severe stress, nausea and debilitation. The onset of these symptoms as well as other manifestations of radiation sickness could be related to the release of certain proteolytic enzymes, called cathepsins, from lysosomes. In order to examine the possibility that lysosomal proteases might be an etiological factor in the acute radiation syndrome, the

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20. ABSTRACT (continued)

levels of cathepsins B1 and D were measured in the serum and spleen homogenates of rats for a period of 22 days following exposure to 1000 rads ~~γ-^{60}Co~~. In addition, β-glucuronidase, an enzyme frequently employed to measure lysosomal enzyme release, was determined. The median level of serum β-glucuronidase was elevated only on day 4; significant decreases occurred on days 1, 2 and 9 through 22. Dramatic elevations in serum cathepsin B1 were observed through most of the investigation. The median cathepsin B1 value in serum was significantly elevated on days 3-6 and 9-15. Splenic β-glucuronidase is increased on day 1, and greatly elevated on days 3-7, after which it declines toward normal values. Splenic cathepsin D rapidly decreased and remained depressed. A biphasic increase in splenic cathepsin B1 on days 1-4 and 10-22 was also observed. The results of this investigation are consistent with the hypothesis that activation and release of lysosomal hydrolases may be an important pathologic event in the later as well as early stages of the acute radiation syndrome. Three possible mechanisms of injury evoked by radiation-induced changes in lysosomal hydrolases are discussed. The results of this study suggest that the release of lysosomal proteases may be a significant factor in radiation injury and stress.

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beta

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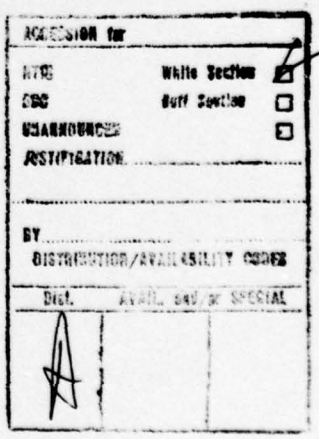

INTRODUCTION

The hypothesis that cell injury and death following radiation exposure may be associated with lysosomal enzyme release was first introduced by Bacq and Alexander.[5] Subsequently, numerous investigators have reported changes in lysosomal membrane permeabilities and hydrolase activities in various tissues following whole-body exposure to ionizing radiation.[1,3,23] Most studies of radiation-induced alterations in lysosomal enzymes have focused on the first 72 hours after exposure. Interest in early changes of lysosomal hydrolases is understandable in view of the possible relationship between enzyme release and primary radiation damage which may result from the intracellular attack of hydrolases on cytoplasmic and nuclear constituents of cells. However, radiation-induced tissue injury and stress could be elicited in several ways. In addition to primary cellular damage, lysosomal hydrolases released from radiation damaged or killed cells may attack previously uninjured cells thereby amplifying initial tissue injury. The action of lysosomal proteases (cathepsins) on serum or tissue proteins can also produce potent inflammatory mediators.[7,15,26,31]

It is conceivable, therefore, that lysosomal hydrolases, especially proteases, may have a profound pathologic effect throughout the acute radiation syndrome. Accordingly the alterations of two lysosomal proteases, cathepsin B1 and D, in the serum and spleen homogenates of rats were determined for a period of 22 days following whole-body exposure to 1000 rads γ-^{60}Co. The levels of β-glucuronidase, an enzyme widely employed to monitor lysosomal enzyme release, were also determined. The potential significance of the perturbations in lysosomal hydrolases observed in this investigation is discussed with reference to mechanisms by which lysosomal hydrolases may intensify tissue injury and stress in the irradiated animal.

METHODS AND MATERIALS

Animals. Male Sprague-Dawley rats (Hla:(SD)) obtained from Hilltop Farms, Scottdale, Pennsylvania, weighing 350-450 g (3- to 4-months-old) were

maintained on a standard laboratory diet (pellets) and allowed access to water ad libitum before and after exposure. Animals were maintained on a 6:00 a.m. light - 6:00 p.m. dark cycle.

Radiation exposure. Rats were placed in Plexiglas restrainers and exposed bilaterally to 1000 rads ^{60}Co (20 rads/min). Six animals were euthanatized by decapitation at each appropriate time interval from 30 min to 22 days postirradiation. The radiation dose used in this study corresponded to an $LD_{65/30}$.

Preparation of homogenates and enzyme assays. Enzyme assays were performed on freshly prepared serum. Spleens were removed, weighed and immediately frozen for later homogenization. Spleen homogenates were prepared as 10 percent (w/v) suspensions in normal saline using an all glass Potter-Elvehjem homogenizer at 0°-4°C. The homogenate was centrifuged at 17,000 x g in a refrigerated RC-2 Sorvall for 20 min. The supernatant fraction was used for the enzyme measurements described in the Results section. This fraction contained 90-100 percent of the total β-glucuronidase activity. All enzyme assays were performed at 37°C using an incubation period of 18 hours. Protein was determined using the method of Lowry et al.[20]

Cathepsin D was measured using a slight modification of the procedure described by Anson.[4] To initiate hydrolysis 0.1 ml of serum or 0.2 ml of a tenfold dilution of the spleen supernatant was added to 2 ml of 1.67 percent hemoglobin in 0.08 citrate at pH 3.0. The reaction was stopped by adding 4 ml of 5 percent trichloroacetic acid (TCA). The enzyme activity is related to the change in absorbance (ΔA) at 280 nm after subtracting the appropriate blank. A change of 1.0 A corresponds to the release of 5.1 μmoles tyrosine.

Cathepsin B1 activity was measured at pH 6.0 in 0.1 M KH_2PO_4-NaOH buffer using α-N-benzoyl-D, L-arginine p-nitroanilide (BPA) as a substrate.[6] To initiate the hydrolysis, 0.2 ml of serum of spleen supernatant fraction, previously activated by incubation (10 min) with 0.1 ml of 20 mM EDTA-20 nM cysteine, was added to 2 ml of BPA reagent. The reaction was stopped by addition

of 1 ml of 17.5 percent TCA. After centrifugation a 1.0-ml portion of supernatant was transferred to a clean tube and the color was developed by addition of 0.2 ml of alkaline stopper reagent.[6] The enzyme activity is reflected in the ΔA at 410 nm. Under conditions of this assay a change of 0.22 A corresponds to the release of 0.10 μmole p-nitroaniline.

β-Glucuronidase activity was measured using phenolphthalein β-glucosiduronate as a substrate.[6] The hydrolysis was initiated by adding 0.2 ml of serum or 20 μl of a tenfold dilution of the spleen supernatant fraction to a mixture of 0.5 ml of 3.0 mM phenolphthalein β-D-glucosiduronate and 0.5 ml 0.3 M acetic acid-NaOH buffer, pH 6.0. The reaction was stopped by addition of 3 ml M Na_2CO_3 and determined at 555 nm. A change of 0.79 A corresponds to the production of 0.10 μmole phenolphthalein.

RESULTS

Serum hydrolase activities. The levels of β-glucuronidase, cathepsin B1 and cathepsin D in rat serum were monitored for a period of 22 days following whole-body exposure to 1000 rads γ-^{60}Co. The results of these investigations are depicted in Figures 1 and 2. Each point represents the activity found in the serum of an individual rat and all experimental observations are shown, except coincidental values. Twelve animals were used to determine the normal range of enzyme activities and, in general, six determinations were performed at each time interval following exposure. Statistical analyses of alterations in lysosomal enzyme activities were based on the median using a two-tailed Mann-Whitney test ($p \leq 0.05$*, 0.01**, 0.002***).[10]

Serum β-glucuronidase. The median value for serum β-glucuronidase in the nonirradiated controls was 1.26×10^{-2} μmoles phenolphthalein released per 0.2 ml serum (Figure 1a). Significant decreases in the median occurred on days 1*, 2*, 8**, 9**, 11***, 13***, 15** and 22**. The values on these days dropped to 70, 70, 10, 24, 19, 49, 30 and 58 percent of the median

control value, respectively. β-Glucuronidase was significantly elevated on day 4* at which time the median had increased by approximately 80 percent.

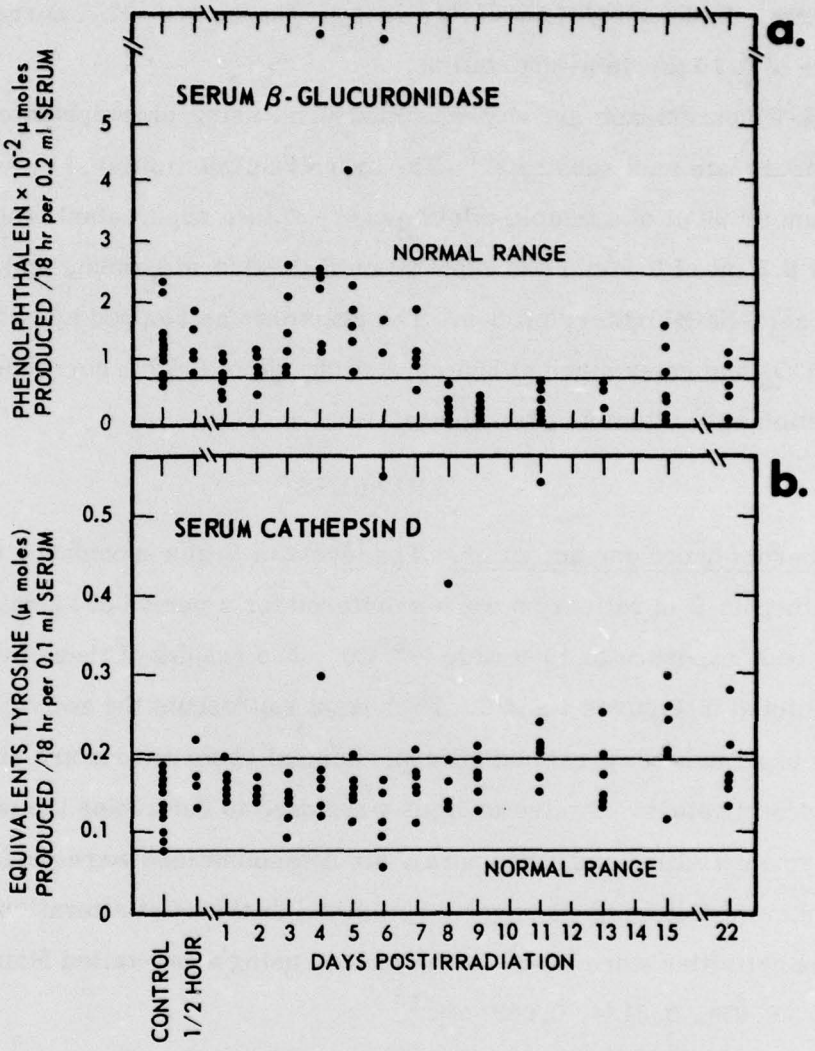

Figure 1. Serum β-glucuronidase (a) and cathepsin D (b) levels of rats exposed to 1000 rads γ-^{60}Co. The region between the solid lines indicates the normal range of values.

Serum cathepsin D. Cathepsin D activity does not appear to be significantly altered in the serum of irradiated rats during the period 0-9 days

(Figure 1b). Significant increases in serum cathepsin D were observed in this study on days 11*, 15** and 22** at which time the median values were elevated 43, 63 and 20 percent above the median control value.

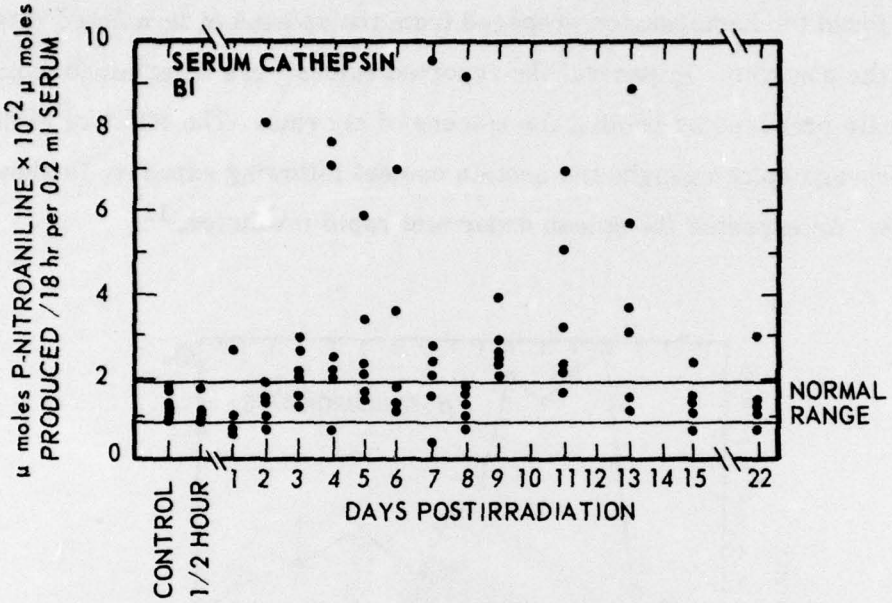

Figure 2. Serum cathepsin B1 levels of rats exposed to 1000 rads γ-^{60}Co. The region between the solid lines indicates the normal range of values.

Serum cathepsin B1. The effect of 1000 rads on serum cathepsin B1 is shown in Figure 2. Rats exposed to ionizing radiation can experience dramatic elevations in serum cathepsin B1 activity. The activities of the nonirradiated controls fell within a relatively narrow range (0.1 - 0.2 μmole p-nitroaniline released per 0.2 ml serum). Following exposure, fivefold to sevenfold increases were common (Figure 2). Significant or highly significant elevations in the median cathepsin B1 levels were observed on days 3**, 4*, 5**, 6*, 9**, 11** and 13**. These corresponded to elevations of 81, 92, 85, 55, 102,

135 and 197 percent respectively. Cathepsin B1 levels appear to return to nearly normal levels at times greater than 15 days.

Spleen hydrolase activities. The effects of irradiation on lysosomal hydrolase activities of 10 percent spleen homogenates are shown in Figure 3. Enzyme activity is expressed as "relative enzyme activity", that is, as the quotient of activity found for homogenates prepared from the spleens of irradiated rats relative to the controls. In general the reported values were determined from a homogenate prepared by pooling the spleens of six rats. The effect of radiation on the average spleen weight and protein content following exposure is shown in Figure 4. As expected the spleen underwent rapid involution.[11]

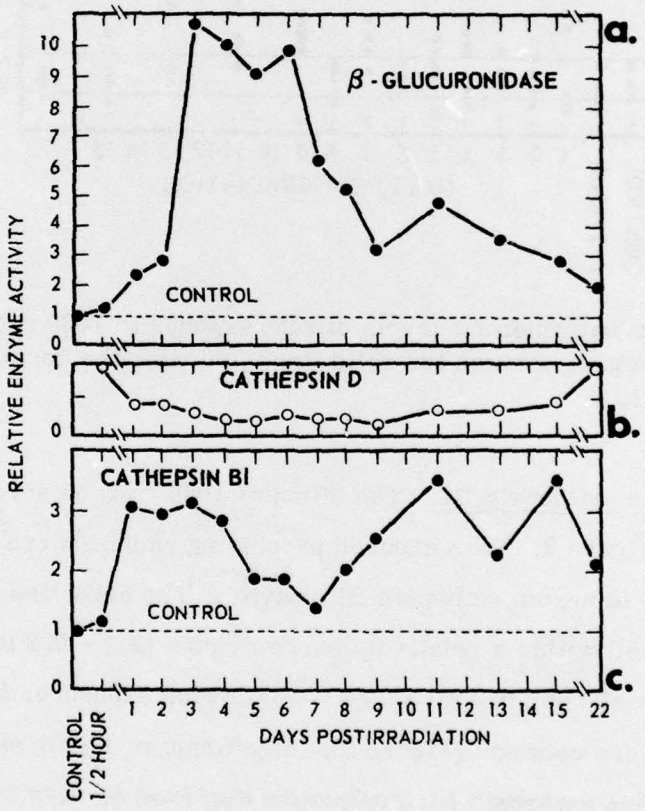

Figure 3. Relative β-glucuronidase (a), cathepsin D (b) and cathepsin B1 (c) activities in homogenates from rats exposed to 1000 rads γ-^{60}Co.

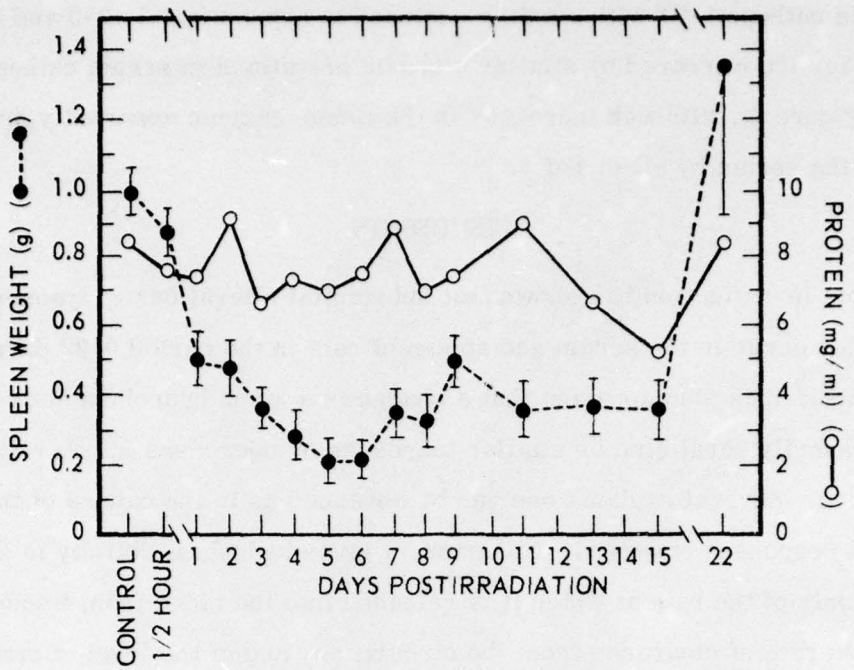

Figure 4. Spleen weight and protein content of 10 percent spleen homogenates of rats exposed to 1000 rads γ-^{60}Co.

Splenic β-glucuronidase. A substantial increase (140 percent) in β-glucuronidase was observed on the 1st day after exposure (Figure 3a). Early activation of specific lysosomal hydrolases in various tissues has been observed by previous investigators.[3,14,16,32] The initial elevation is followed by a very pronounced increase at 3-7 days.

Splenic cathepsin D. In sharp contrast to β-glucuronidase, cathepsin D activity in spleen decreased rapidly and remained depressed throughout the course of this investigation (Figure 3b). The striking similarity of changes in spleen weight (Figure 4) and cathepsin D activity (Figure 3b) supports the conclusion[8,9] that cathepsin D is associated specifically with lymphocyte lysosomes in the spleen.

Splenic cathepsin B1. Cathepsin B1 activity in the spleen is greatly enhanced 1 day after irradiation. An increase in cathepsin B on day 1 post-irradiation has also been reported by Bouma and Gruber.[8] Biphasic elevations

in splenic cathepsin B1 with maxima centered at approximately 2-3 and 13 days (Figure 3c) are mirrored by similar biphasic elevations in serum cathepsin B1 levels (Figure 2), although increases in the tissue enzyme apparently precede those in the serum by about 1 day.

DISCUSSION

This investigation has shown that substantial alterations in lysosomal hydrolases occur in the serum and spleen of rats in the period 0-22 days post-irradiation. It is also apparent that a change in a given hydrolase activity is not necessarily paralleled by similar increases or decreases in other lysosomal hydrolases. Several explanations can be advanced as to the causes of the differential responses observed. In serum, a given hydrolase activity is a function not only of the rate at which it is released into the blood from tissues, but also of its rate of clearance from the circulation, and of the level of circulating inhibitors. Similarly changes in the tissue levels of hydrolases are influenced by changes in their relative rates of synthesis and breakdown; inhibitors; changes in relative cell populations (see for example, splenic cathepsin D, Results section); and rates of loss due to changes in membrane permeability. Further, it should be noted also that the responses observed in this study, particularly those occurring at longer times after irradiation, may not be the direct result of radiation damage per se, but may reflect metabolic alterations that occur as a consequence of the compromised immune status of the animal (e.g., damaged lymphocytes, infection, endotoxemia).

The early fall in serum β-glucuronidase seen in this investigation agrees with the decreases reported in β-glucuronidase and acid phosphatase activities found in the serum and urine of rats 1-2 days after irradiation.[2,18] We are aware of no other studies measuring changes in typical lysosomal hydrolases for an extended period following radiation exposure. However the response of β-glucuronidase (Figure 1a) is very similar to changes in p-nitrophenyl acetate esterase activity in the serum of irradiated rats.[27] The depressed serum

β-glucuronidase from 7-22 days postirradiation is probably the result of leukopenia in this period.[11] The effect of radiation on splenic β-glucuronidase activity is similar to that reported for acid phosphatase in macrophage lysosomes of irradiated mice.[23]

The finding that serum cathepsin D in rats remains unaltered 0-48 hours postirradiation is in opposition to the results of Vranovska et al.[29] These earlier workers reported a decrease in serum protease activity (pH 3.8) of rats 1 day after exposure to 800 rads. The marked decrease in cathepsin D activity found in homogenates prepared from the spleens of rats 1 day after exposure is in complete accord with the previous observations of Bouma and Gruber.[8]

In an earlier study Koćmierska-Grodzka[17] found no significant elevation in cathepsin B in the serum of rats 0-3 days after exposure to 800 rads. Since the first significant elevation in the median serum cathepsin B1 value in this study was observed on day 3, using a slightly higher dose (1000 versus 800 rads), the results of this study cannot be considered to be inconsistent with those of Koćmierska-Grodzka. Moreover the increase in splenic cathepsin B1 is consistent with the results of the previous investigator.[17]

Early increases in lysosomal hydrolases have been reported[16,32] and the early increases of splenic β-glucuronidase and cathepsin B1 observed in this study (Figure 3) are consistent with the notion that the lysosome may be one of several primary sites of radiation cell damage. Sequestered with the lysosome are hydrolytic enzymes capable of digesting virtually every cell constituent. Radiation causes increased lysosomal membrane permeability,[3] activation of hydrolases[23,24] and increased numbers of lysosomes.[24] It is probable that release of these hydrolases from the lysosome could result in direct intracellular attack on surrounding cytoplasmic and/or nuclear contents thereby disrupting normal cellular metabolic and mitotic processes. Although direct damage to the nucleus is generally considered to be the primary event in radiation cell damage (see, for example reference 11, Chapter 5), a mechanism involving prior activation and release of lysosomal hydrolases followed by an intracellular

attack on the nucleoplasm cannot be disregarded.[24,25] In support of this contention, Paris and Wang[22] found that immunologic inhibition of lysosomal enzymes increased survival in irradiated CCL 47 rat sarcoma cell cultures even though the antilysosomal antibodies were cytotoxic.

When cells are killed or damaged by radiation, regardless of mechanism, lysosomal hydrolases may then be released into the intercellular milieu. This is a potential mechanism for amplifying tissue injury, since hydrolytic enzymes may then attack previously uninjured cells. Evidence for such a process termed "lysosomal cytolysis chain reactions" has recently been summarized.[28] Such a mechanism may be particularly important in tissues having a pH conducive to the release of enzymes from the lysosome[28] or those normally rich in lysosomal hydrolases (e.g., spleen).[8]

The changes in the three hydrolases of spleen in the first few days post-irradiation (Figure 3) of this study are consistent with a lysosomal cytolysis chain reaction. During the first 24 hours after irradiation a rapid cytolysis of the radiosensitive lymphocytes occurs.[1] The destruction of lymphocytes is reflected both in the decrease in spleen weight (Figure 4) and cathepsin D activity (Figure 3b).[8,11] Lymphocytolysis could initiate the lysosomal cytolysis chain reaction since extracellularly released hydrolases (or other lymphocyte factors) may then attack previously uninjured macrophages.

Lysosomal proteases and the peptide mediators they produce are pathologic agents in inflammation,[31] circulatory disorders[7] and shock.[7,15] It is not surprising, therefore, that proteases and kinins are recognized as potential radiotoxic substances.[17,19] Protease inhibitors have been found to have radioprotective properties.[13,19] Increases in serum and tissue kinins in the irradiated animal could result in increased vascular and tissue permeability, impaired tissue perfusion, acidosis, and ischemia.

Several investigators have studied early changes in tissue[8,13,17,18] and serum[17,29] lysosomal proteases following whole-body exposure. Unfortunately, detailed information regarding the responses of lysosomal proteases in midline

lethally irradiated animals in the later stages (5-30 days postirradiation) of the acute radiation syndrome has been lacking. This investigation has shown that substantial elevations occur in cathepsin B1, a major lysosomal protease, not only early in radiation sickness but also in the later stages. Moreover, elevations in splenic and serum cathepsin B1 are biphasic and changes in tissue levels apparently precede those in the serum. This suggests that the increased serum cathepsin B1 levels may be reflecting leakage of this enzyme from radiosensitive tissues. The exact origin of serum cathepsin B1 is unknown; however, experiments in this laboratory have shown that circulating granulocytes and lymphocytes contain little, if any, cathepsin B1. The serum levels of cathepsin D are either unaffected (0-10 days) or only modestly increased later on (11, 15 and 22 days), while splenic levels are substantially decreased. Cathepsin B1 may be an important inflammatory factor in the radiation syndrome since this enzyme can generate bradykinin and other toxic peptides directly.[15] Alternatively, it may act indirectly by activating other proteolytic enzymes which produce toxic factors, trigger coagulative, fibrinolytic or complement cascades.[21] Indeed, if increased levels of cathepsin B1 (or other proteases) evoke concomitant increases in tissue or serum kinins, then one might expect a breakdown in normal biological barriers. In this regard, it is intriguing that a biphasic endotoxemia in irradiated mice has been reported[30] very like the cathepsin B1 response observed in this study. There is a possibility that the leakage of endotoxin from the lumen of irradiated animals may be associated with increased amounts of kinins generated by lysosomal proteases. The possible connection between lysosomal protease activation and the incidence of endotoxemia (or bacteremia) is further strengthened by the observations of Creasey et al.[12] These investigators found a transient radiation-induced increase in capillary permeability in rabbit skin occurring on day 2, followed by a sustained elevation on days 10 through 17 postirradiation.

In summary, perturbations of lysosomal hydrolases are present throughout the radiation syndrome. These enzymes may contribute to the death of

radiosensitive cells. They may also contribute to the injury and death of non-radiosensitive tissues via lysosomal cytolysis chain reactions. Finally, leakage of lysosomal proteases into the systemic circulation may release factors or trigger inflammatory cascades causing a breakdown in homeostasis ultimately resulting in death. Control of these enzymes may be of benefit in treating radiation injury.

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